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**Sent:** Thursday, May 16, 2002 2:41 PM  
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please locate the following:

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Thanks,  
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- position -35; an Alu I-Bam HI fragment from pML(C<sub>2</sub>AT) (9), consisting of 377 bp of G-minus sequence, followed by 5 bp of Sma I and Bam HI recognition sequences. The UAS fragment in pGALACG- was derived from pCZGAL (15) and contained a single GAL4-binding site. The UAS fragment in p(DED48)<sup>2</sup>CG- was derived from pCZ(DED48)<sup>2</sup> (A. R. Fuchman and R. D. Kornberg, in preparation) and contained two copies of the T-rich element from upstream of the DED1 gene. Removal of the GAL4-binding site from pGALACG- by cleavage with Xba I and Eco RI, followed by filling in with the large fragment of DNA polymerase I and ligation, gave pACG-.
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  22. We thank A. Edwards and R. Kellcher for help in developing the assay for initiation in yeast extracts with G-minus templates. We thank R. Roeder for gifts of pML(C<sub>2</sub>AT) and p(C<sub>2</sub>AT)19, and R. Tjian for a gift of HeLa nuclear extract. K.S. received partial support from the Toyobo Biotechnology Foundation. Costs of this research were paid from NIH grant GM36659 (R.D.K.).

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## Identification of an AUUUA-Specific Messenger RNA Binding Protein

JAMES S. MALTER

An important control point in gene expression is at the level of messenger RNA (mRNA) stability. The mRNAs of certain regulatory cellular proteins such as oncogenes, cytokines, lymphokines, and transcriptional activators are extremely labile. These messages share a common AUUUA pentamer in their 3' untranslated region, which confers cytoplasmic instability. A cytosolic protein was identified that binds specifically to RNA molecules containing four reiterations of the AUUUA structural element. This protein consists of three subunits and binds rapidly to AUUUA-containing RNA. Such protein-RNA complexes are resistant to the actions of denaturing and reducing agents, demonstrating very stable binding. The time course, stability, and specificity of the protein-AUUUA interaction suggests the possibility that the formation of this complex may target susceptible mRNA for rapid cytoplasmic degradation.

**D**ESPITE GREAT PROGRESS IN elucidating the mechanisms of transcriptional regulation of gene expression, relatively little is known about post-transcriptional control at the level of mRNA turnover (1). In a wide variety of organisms and cell types, mRNAs display heterogeneous cytoplasmic stability (1). Inducible growth regulators such as oncogene products (2), cytokines (3), and transcriptional activators (4) tend to have extremely unstable messages with half-lives on the order of 10 to 30 min. Treatment of cells with phorbol esters (5), antibodies to cell surface proteins (6), serum (4), or protein synthesis inhibitors such as cycloheximide (6, 7) can modulate the half-lives of rapidly degraded messages. Therefore, the rates and selectivity of mRNA degradation are variable, and regulation of these processes are important control points of gene expression.

The mechanisms by which mRNA is targeted for rapid turnover are poorly understood (1). A recurrent motif of rapidly degraded mRNA is an AU-rich structure in the 3' untranslated region (1, 8). In particular, the pentamer AUUUA is present singly or in multiple reiterations in a wide variety of oncogene and cytokine mRNA 3' untranslated regions. Removal of this region confers significantly greater stability to messages produced from transfected constructs (2, 7, 8), whereas the addition of a short DNA segment coding for this motif destabilized previously stable messages (8). Thus it has been proposed that a trans factor may recognize this AU-rich motif and in some way target susceptible mRNA for degradation (1, 8).

To determine if a cytoplasmic protein (or proteins) specifically interacts with the 3' untranslated region of unstable mRNA through the AUUUA element, we incubated lymphocyte cytoplasmic extract with *in vitro* transcribed, labeled RNA that con-

**Fig. 1.** Detection of protein-RNA complexes by band-shift assay. Cytoplasmic extracts of Jurkat cells were prepared by freeze-thaw lysis in 25 mM tris-HCl (pH 7.9), 0.5 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride, followed by centrifugation at 15,000g at 4°C for 15 min. RNAs were transcribed by T7 RNA polymerase and labeled with [<sup>32</sup>P]UTP (uridine triphosphate) to a specific activity of 10<sup>7</sup> cpm per microgram of RNA from Eco RI-digested pT7/T3-α19 (60-base nonspecific probe) (BRL) or Kpn I-digested pT7/T3-α19-AUUUA [64-base specific probe with four AUUUA repeats (16); coding oligonucleotides were cloned into the unique Sma I site]. Cytoplasmic extract (from 2 × 10<sup>7</sup> cells) was incubated with 10<sup>4</sup> cpm of RNA (0.5 to 1 ng), in 10% glycerol, 12 mM Hepes (pH 7.9), 15 mM KCl, 0.25 mM EDTA, 0.25 mM dithiothreitol, 5 mM MgCl<sub>2</sub>, and *Escherichia coli* transfer RNA (200 ng/μl) in a total volume of 10 μl for 10 min at 30°C. RNase A was added to a final concentration of 1 μg/μl, and reaction mixtures were incubated for 30 min at 37°C before electrophoresis in a 7% native polyacrylamide gel with 0.25× TBE running buffer (10). Lane 1, AUUUA-containing specific probe alone; lane 2, specific probe and lysate; lane 3, nonspecific probe alone; and lane 4, nonspecific probe and lysate. The positions of complexed and free probe are indicated.



tained four adjacent reiterations of the AUUUA motif. After a brief incubation, the reaction mix was treated with ribonuclease A (RNase A) and the presence of protected complexes then assessed by band-shift assay on native, low ionic strength polyacrylamide gels (Fig. 1). A stable, RNase A-resistant complex was detected. Neither a control RNA probe of similar size, nucleotide content, and specific activity but lacking the AUUUA reiterations (Fig. 1) nor an RNA probe of similar size containing four UAAAU repeats formed stable complexes (9). The complex formed with the probe containing the AUUUA motifs was stable in the presence of RNase A for at least 2 hours (9) or when resolved in higher ionic strength running buffers such as 0.5× or 1× tris-borate-EDTA (TBE) (9, 10). Complex formation was abolished by prior incubation of the lysate with proteinase K (2.5 mg/ml) for 15 min (9).

The molecular size of the complex was assessed after ultraviolet (UV) light-induced cross-linking of the lysate-probe reaction mixture and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A). A stable complex migrating with a molecular mass of 28 to 45 kD was observed with the AUUUA-containing probe, but not the control probe, and stable complexes were not observed when specific or nonspecific

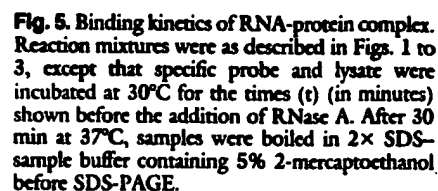
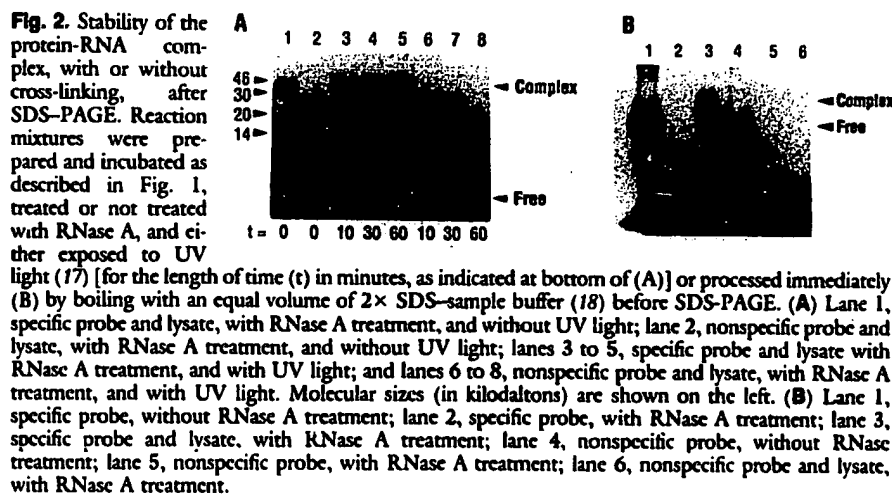


Fig. 5. Binding kinetics of RNA-protein complex. Reaction mixtures were as described in Figs. 1 to 3, except that specific probe and lysate were incubated at 30°C for the times (t) (in minutes) shown before the addition of RNase A. After 30 min at 37°C, samples were boiled in 2× SDS-sample buffer containing 5% 2-mercaptoethanol before SDS-PAGE.

ing AUBP does not effectively recognize poly U tracts.

To assess the kinetics of interaction between the AUUUA-containing probe and cytoplasmic binding activity, I incubated the probe with lysate for various times before the addition of RNase A (Fig. 5). Incubations as short as 1 min yielded the same amount of complex as 1-hour incubations. When lysate, probe, and RNase A were added simultaneously, all three subunits were detected, although with decreased intensity (Fig. 5). Thus, stable complex formation appears to occur rapidly and likely precedes exonuclease-mediated shortening of polyadenylated tails that appears to be the primary degradative event of labile mRNA (7, 13).

The number of reiterations or which regions of the AUUUA pentamer are required for the stable, specific binding observed here is not known, nor is molecular mechanism of AUBF-RNA interaction. Active site sulfhydryls of the iron response element-binding protein have been implicated as the mediators of binding to the iron response element in ferritin mRNA (14). The stability of the AUBF-RNA complex is consistent with such a mechanism. In addition, because the mRNA degradative system can discriminate between different AU-rich mRNAs (15), it is possible that AUBF is a member of a family of proteins with multiple domains for recognition of the common AUUUA motif as well as other message-specific elements.

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Fig. 3. The effect of denaturation and disulfide bond reduction on stability of the protein-RNA complex with or without UV cross-linking. Reaction mixtures were prepared and incubated as described in Fig. 1, but boiled in an equal volume of 2× SDS-sample buffer containing 5% 2-mercaptoethanol before SDS-PAGE. Time of exposure to UV light (t) is in minutes; nonspec., nonspecific. Molecular sizes (in kilodaltons) are shown on the left.

probes and RNase A were incubated alone (Fig. 2B). Unexpectedly, stable complexes were consistently found in reaction mixtures immediately subjected to electrophoresis without exposure to UV light (Fig. 2B), suggesting that the UV treatment was not required for complex stability.

Reduction of the lysate-probe mixture with 2-mercaptoethanol followed by SDS-PAGE resolved the complex into three components of approximately 15, 17, and 19 kD (Fig. 3). These data suggest that each of the protein subunits has a binding site for the AUUUA sequence. Because dissociation of the binding protein [herein denoted adenosine-uridine binding factor (AUBF)] occurred after treatment with reducing agents, the subunits are likely held together by interchain disulfide linkages. The stability of the complex under denaturing or reducing conditions is presumably the result of highly specific, hydrogen-bond interactions between AUBF and RNA. My observations are consistent with the high-affinity RNA-binding characteristics of a family of heterogeneous nuclear ribonucleoproteins (11). It

Fig. 4. Inhibition of formation of the RNA-protein complex by competitor RNA. Reaction mixtures were as described in Fig. 1, except that various amounts of unlabeled specific or nonspecific RNA were incubated with the cytoplasmic lysate for 10 min at 30°C before the addition of labeled, specific probe for 10 min. After RNase A treatment for 30 min, all samples were exposed to UV light for 20 min at 0°C and boiled in 2× SDS-sample buffer containing 5% 2-mercaptoethanol. Lane 1, specific probe and lysate, without unlabeled competitor RNA; lane 2, nonspecific probe and lysate, without unlabeled competitor; lanes 3 to 8, specific probe and lysate in the presence of increasing molar excess (shown) of unlabeled AUUUA RNA or control, nonspecific RNA.

is not known whether the subunits represent dissimilar or related peptides, as variable nuclease protection of the probe by individual subunits could account for the small observed differences in complex molecular mass (12).

The specificity of the AUBF-RNA interaction was assessed by competition experiments. Lysate was incubated with increasing amounts of unlabeled AUUUA-containing probe or control RNA before incubation with labeled AUUUA-containing probe (Fig. 4). The unlabeled specific probe effectively competed with its labeled analog for binding to the three protein subunits. A 100-fold excess of homoribonucleotide polymers [poly(A) or poly(U)] or coribonucleotide polymers [poly(A, U), poly(A, C, U), or poly(A, G, U)] did not significantly decrease specific probe binding (9), indicat-

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